Amendments to the Specification

Please replace lines 16-22 on page 5 with the following:

Figure 3 is a process flow diagram for fermentation and ~~Streamline~~ STREAMLINE® SP chromatography (cation-exchange resin) for angiostatin production.

Figure 4 is a process flow diagram for chromatography steps following ~~Streamline~~ STREAMLINE® SP chromatography (cation-exchange resin) for angiostatin production.

Figure 5 is a process flow diagram for chromatography, ultrafiltration, diafiltration and formulated bulk processing steps following ~~Toyopearl~~ TOYOPEARL® (hydrophobic interaction resin) chromatography for angiostatin production.

Please replace line 390 on page 18 to line 410 on page 19 with the following:

The first chromatography step in the recovery and purification procedure is called the angiostatin protein purification capture step, and the specific resin used is called ~~Streamline~~ STREAMLINE®-SP (Pharmacia, Inc.). SP refers to the sulfopropyl functional groups that are attached to the support bead that give the resin its cationic character. It is to be understood that besides ~~Streamline~~ STREAMLINE®-SP resin, other resins that act as cation exchangers may be used in the practice of the present invention. Such cation exchangers include but are not limited to carboxymethylcellulose. ~~Streamline~~ STREAMLINE® refers to a relatively new format of chromatography that is designed to capture and separate target protein from a milieu of broth, thus eliminating the need for centrifugation to separate cells from the protein-containing supernatant. This type of chromatography is also known as expanded bed absorption chromatography (EBA). In practice, the broth is typically pumped up into a ~~Streamline~~ STREAMLINE® column containing about 20-30% by volume of settled resin and approximately 70-80% buffer. As the broth enters the column, the bed of resin expends and flows up, thereby accounting for the name

EBA. As the bed flows up, protein is bound to the beads, which can only flow up a finite distance, to an equilibrium level. The cells and non-bound protein however, flow up and out of the column to waste. Once all the broth has been pumped onto and traversed the column, the flow direction is reversed (now in the downward direction) and the resin is allowed to pack. What remains is a functional column that can be washed and eluted in the more conventional sense. Angiostatin protein is eluted from this column with salt, and is ready for the next chromatographic step.

Please replace lines 413-415 on page 19 with the following:

The next chromatographic steps in the process are the Q-~~Sepharose~~ SEPHAROSE® (ion exchange) and hydroxyapatite chromatography columns. These steps are followed by the phenyl column as shown in the figures.

Please replace line 430 on page 19 to line 435 on page 20 with the following:

~~Streamline~~ STREAMLINE® SP Chromatography (cation-exchange resin)

The expanded bed column (60cm x 18cm (settled height), 51L of ~~Streamline~~ STREAMLINE® SP Resin, expanded bed volume ~ 150L, expanded bed height of ~54cm at 300 cm/hr) is sanitized with 0.5M NaOH (held for a minimum of 6 hours). The column is rinsed with WPU until neutral conditions are met.

Please replace line 453 on page 20 to line 465 on page 21 with the following:

Q ~~Sepharose~~ SEPHAROSE® FF and Ceramic Hydroxyapatite Chromatography

The Q-~~Sepharose~~ SEPHAROSE® (ion exchange) column (30cm x 15cm column, 10.61, CV) and Ceramic Hydroxyapatite (CHT) column (45cm x 37cm column, 58.8L CV) which were stored in 0.1M NaOH are rinsed with 5 CV's of 10mM Sodium Phosphate, pH 7.0. The maximum flowrate for this chromatography is 480LPH (300 cm/hr of CHT Column) and is performed at ambient temperature. The angiostatin flows through the Q ~~Sepharose~~ SEPHAROSE® (ion exchange) column and binds to the CHT column. The columns are charged with 0.5M Sodium Phosphate, pH 7.0 then equilibrated with 10mM Sodium Phosphate, pH 7.0 until the pH and conductivity are that of the equilibration buffer. The elution from the ~~Streamline~~

STREAMLINE® SP Chromatography of Angiostatin is diluted inline with WFI (1 part elution: 3 part WFI) and loaded onto the column. The column is washed to baseline with 10mM Sodium Phosphate, pH 7.0. The Q ~~Sepharose~~ SEPHAROSE® (ion exchange) column is removed from the chromatography skid.

Please replace lines 473-477 on page 21 with the following:

The CHT column is regenerated with 0.5M Sodium Phosphate, pH 7.0. The Q ~~Sepharose~~ SEPHAROSE® FF column is regenerated with 2M NaCl. The columns are then cleaned with 0.5M NaOH and held for at least 1 hour (maximum of 24 hours). The columns are then stored in 0.1M NaOH which is prepared by blending 0.5M NaOH and WFI.

Please replace lines 478-481 on page 21 with the following:

~~Toyopearl~~ TOYOPEARL® Phenyl 650M Chromatography

The ~~Toyopearl~~ TOYOPEARL® Phenyl 650M (hydrophobic interaction resin) Column (45cm x 25cm column, 40L CV) which was stored in 0.1M NaOH is rinsed with WFI until neutral conditions have been met. The flowrate for this chromatography is 480LPH (300cm/hr) and is performed at ambient temperature. The column is equilibrated with 50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1 until the pH and conductivity are that of the equilibration buffer.

Please replace line 485 on page 21 to line 501 on page 22 with the following:

The elution from the CHT column is diluted inline with 50mM Sodium Phosphate, 24mM Citric Acid, 2.8M Ammonium Sulfate, pH 4.5 (1 part elution: 1 part buffer) and loaded onto the ~~Toyopearl~~ TOYOPEARL® 650M column. The loaded column is then washed with 50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1. The angiostatin is eluted from the column using a 20CV linear gradient from 50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1 to 50mM Sodium Phosphate, 24mM Citric Acid, 0.92M Ammonium Sulfate, pH 5.1. The 50mM Sodium Phosphate, 24mM Citric Acid, 0.92M Ammonium Sulfate is continued until UV returns to < 0.1 AU. The eluate is collected from peak

beginning at 0.3 AU to peak ending at 0.1 AU. The elution volume is approximately 8CV's at an angiostatin concentration of ~ 0.7g/L. The column is regenerated with 50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1. The column is rinsed with WFI and then cleaned with 0.5M NaOH. The column is then stored in 0.1M NaOH which may be prepared by blending 0.5M NaOH inline with WFI. Note: If the ~~Toyopearl~~ TOYOPEARL® Phenyl Elution will not be processed within 8 hours, the elution is to be diluted lx with WFI and stored at 2-8°C for a maximum of 48 hours.

Please replace lines 505-518 on page 22 with the following:

100sq. feet of 5Kd polyethersulfone filters are sanitized with 0.5M NaOH and held in 0.5M NaOH for a minimum of 1 hour (maximum of 2 hours). The filters are then rinsed with WFI until neutral conditions are obtained. The filters are then equilibrated with 0.15M Sodium Chloride until the retentate pH and conductivity is that of the equilibration buffer. The ~~Toyopearl~~ TOYOPEARL® Elution (if not diluted) is diafiltered 1x with 0.15M Sodium Chloride. The Diafiltered product is concentrated to 5 mg/mL then diafiltered again until the pH and conductivity is that of the formulation buffer (~7 volumes). The UF/DF skid is rinsed with 2 x 10L flushes which are added to the diafiltered product. Due to the hold up volume of the UF/DF skid, it is necessary to perform the final concentration on a table top unit with 25sq. feet of filter. The retentate is then concentrated to 20.0 mg/mL. The UF/DF filters are rinsed with 0.15M Sodium Chloride and the rinse is added to the concentrated product. The UF/DF retentate is adjusted with 0.15M Sodium Chloride to a final concentration of 15 mg/ml. Note: If the ~~Toyopearl~~ TOYOPEARL® Elution was diluted the lx diafiltration may be omitted.

Please replace page 25 with the following:

~~Streamline~~ STREAMLINE® SP Chromatography (cation-exchange resin)

Column

Specifications

Resin:	~~Streamline~~ STREAMLINE® SP
Type:	Expanded Bed Adsorption
Particle Size:	200micron
Dimensions:	60cm x 18cm, 51L Resin, expanded bed volume ~ 150L, expanded bed height of ~54cm at 848 LPH
Pressure:	2.0 bar
Limitation:	
Expected Flowrate:	848 LPH (660 - 740 LPH for load and wash)

Sanitization and Rinse

Buffer:	0.5M NaOH
Approximate Volume Required	7CV (357L)
Flow Direction:	Up
Hold Time:	Minimum of 6 hours (maximum of 24 hours)
WPU Rinse:	Until conductivity < 1.0 mS/cm

Equilibration

Buffer:	50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1
Approximate Volume Required:	14CV (714L)
Flow Direction:	Up
Equilibration Specifics:	Conductivity 5.5 - 6.5mS/cm PH = 5.1 ± 0.2

Please replace page 28 with the following:

Purification

General Purification

Storage Conditions:	Storage >8 hours at 2-8°C No stability data has been generated. Therefore, storage time should be limited to less than 24 hours. Final product storage is -70°C,
Extinction Coefficient:	2.08
Shear Sensitivity:	Not Determined
Concentration Limit:	Not Determined
In process Testing	LAL, UV, PD and QC retains

Q ~~Sepharose~~ <u>SEPHAROSE®</u> <u>(ion exchange)</u> and Ceramic Hydroxyapatite Chromatography

Column Specifications

Resin:	Q ~~Sepharose~~ <u>SEPHAROSE®</u> FF (Pharmacia)
Type:	Ion Exchange (Flowthrough)
Particle Size:	90micron
Dimensions:	30 cm D x 15 cm H 1 O.GL CV
Pressure Limitation:	3.0 bar
Expected Flowrate:	480 LPH
Resin:	Ceramic Hydroxyapatite (Biorad)
Type:	Mixed Mode
Particle Size:	40micron
Dimensions:	45 cm D x 37 cm H 58L CV
Pressure Limitation:	2.5 bar
Expected Flowrate:	480 LPH

Rinse

Buffer:	l0mM Sodium Phosphate, pH 7.0
Specifics:	Rinse until conductivity < 3.0 mS/cm

Please replace page 29 with the following:
Charge

Buffer:	O.5M Sodium Phosphate, pH 7.0
Approximate Volume Required:	3CV (175L)
Flow Direction:	Up

Equilibration

Buffer:	l0mM Sodium Phosphate, pH 7.0
Approximate Volume Required:	5 - 7CV (292 - 408L)
Flow Direction:	Up
Equilibration Specifics:	pH = 7.0 ± 0.1 Conductivity =1.0 - 1.6 mS/cm

Load

Capacity:	6 - 13 mg/mL
Conductivity:	4 - 6 mS/cm
WFI Dilution:	3 Volumes Inline
Flow Direction:	Up
Loading Time:	1.5 hr
Volume of Load:	600L (4x ~~Streamline~~ STREAMLINE® Elution)

Wash

Buffer:	l0mM Sodium Phosphate, pH 7.0
Approximate Volume Required:	3CV (175L)
Flow Direction:	Up
Wash Specifics:	pH = 7.0 ± 0.2

Elution (from CHT)

Type:	Linear Gradient 0 to 100% (A to B)
Buffer A:	10mM Sodium Phosphate, pH 7.0
Buffer B:	74mM Sodium Phosphate, pH 7.0
Approximate Volume Required:	SCV (239L) Hold in B for SCV (239L)
Flow Direction:	Up
Product Collection:	Start @ 0.15 AU pre Peak and end @ 0.3 AU post Peak
Elution Specifics: .	pH = 7.0 ± 0.2 Volume - 4-S CV Angiostatin® concentration ~ 0.8 mg/mL

Please replace page 30 with the following:

Regeneration of CHT Column

Buffer:	O.5M Sodium Phosphate, pH 7.0
Approximate Volume Required:	3CV (143L)
Flow Direction:	Down

Regeneration of Q ~~Sepharose~~ SEPHAROSE® Column

Buffer:	2M NaCI
Approximate Volume Required:	3CV (32L)
Flow Direction:	Down

Cleaning (both columns)

Buffer:	O.5M NaOH
Approximate Volume Required:	SCV (292L)
Flow Direction:	Down
Hold Time:	Minimum 1 hour (Maximum of 24 hours)

Storage

Buffer:	0.1M NaOH
Approximate Volume Required:	3CV (175L)
Flow Direction:	Down

Please replace page 31 with the following:

~~Toyopearl~~ **TOYOPEARL® Phenyl 650M Chromatography**

~~Toyopearl~~ **TOYOPEARL® Phenyl 650M Column (hydrophobic interaction resin)**

Specifications

Resin:	~~Toyopearl~~ TOYOPEARL® Phenyl 650M (TosoHaas)
Type:	Hydrophobic Interaction
Particle Size:	65micron
Dimensions:	45 cm D x 25 cm H 40L CV
Pressure Limitation:	2.5 bar
Expected Flowrate:	480 LPH

Rinse

WFI Rinse:	Until conductivity < 1.0 MS/cm
Rinse Specifics:	Perform a 3CV gradient from O.1M NaOH to WFI then continue rinsing
Flow Direction:	Up

Equilibration

Buffer:	50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1
Transition Specific:	Perform a 3CV gradient from WFI to EQ buffer the continue with equilibration
Approximate Volume Required:	4.5CV (180L)
Flow Direction:	Up
Equilibration Conductivity 154 - 171 mS/cm	
Specifics:	Density= 1.10 Kg/L

Load

Capacity:	> 12 mg/mL
Conductivity:	154 - 171 mS/cm
Buffer Dilution:	lx (50mM Sodium Phosphate, 24mM Citric Acid, 2.8M Ammonium Sulfate, pH 4.6)
Volume Buffer Required:	~ 350L
Flow Direction:	Up
Loading Time:	1.25 hr
Volume of Load:	~ 700L

Please replace page 36 with the following:

Buffer *Preparation*

General Buffer Preparation

Specifics:	Buffers made by volume 21 day expiration on all buffers (based on safety)
In Process Testing:	pH and Conductivity (measured @ 18-22°C) Density for ~~Streamline~~ STREAMLINE® Wash Buffer and Phenyl Buffers
QC Testing:	LAL and Bioburden

50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1

Unit Description:	Op ~~Streamline~~ STREAMLINE® Equilibration and ~~Toyopearl~~ TOYOPEARL® 650M Regeneration	
Component and Concentration:	Sodium Phosphate, Dibasic	13.4 g/L
	Citric Acid, Monohydrate	5.04 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	5 - 7 mS/cm	
pH:	4.9 - 5.3	
Density:	1.00 Kg/L	

30mM Sodium Phosphate, 200mM NaCl, pH 7.2

Unit Description:	Op ~~Streamline~~ STREAMLINE® Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	6.40 g/L
	Sodium Phosphate, Monobasic	0.846 g/L
	Sodium Chloride	11.69 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	19-23mS/cm	
pH:	7.0 - 7.4	
Density:	1.00 Kg/L	

15% Glycerol, 15mM Sodium Phosphate, pH 6.1

Unit Description:	Op ~~Streamline~~ STREAMLINE® Wash	
Component and Concentration:	Glycerol	189 g/L (15% v/v)
	Sodium Phosphate, Dibasic	0.7 g/L
	Sodium Phosphate, Monobasic	2.08 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	< 1.5 mS/cm	
pH:	5.9 - 6.3	
Density:	~ 1.04 Kg/L	

Please replace page 37 with the following:

10mM Sodium Phosphate, pH 7.0

Unit Description:	Op Q ~~Sepharose~~ SEPHAROSE® and CHT Equilibration/Wash/Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	1.63 g/L
	Sodium Phosphate, Monobasic	0.54 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	1.0 - 1.6 mS/cm	
pH:	6.9 - 7.1	
Density:	1.00 Kg/L	

74mM Sodium Phosphate, pH 7.0

Unit Description:	Op Q ~~Sepharose~~ SEPHAROSE® and CHT Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	12.1 g/L
	Sodium Phosphate, Monobasic	3.98 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	6.0 - 7.2 mS/cm	
pH:	6.9 - 7.1	
Density:	1.00 Kg/L	

0.5M Sodium Phosphate, pH 7.0

Unit Description:	Op Q ~~Sepharose~~ SEPHAROSE® and CHT Regeneration	
Component and Concentration:	Sodium Phosphate, Dibasic	81.8 g/L
	Sodium Phosphate, Monobasic	26.9 g/L
pH Adjustment:	NaOH or Phosphoric Acid	TBD
Conductivity:	35 - 41 mS/cm @ 18-20°C	
pH:	6.9 - 7.1	
Density:	1.00 Kg/L	

Please replace page 38 with the following:

50mM Sodium Phosphate, 24mM Citric Acid,

1.4M Ammonium Sulfate, pH S._1

Unit Description:	Op ~~Toyopearl~~ TOYOPEARL® 650M Equilibration/Wash/Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	13.4 g/L
	Citric Acid	5.04 g/L
	Ammonium Sulfate	185.0 g/L
pH Adjustment:	NaOH or HCl	
Conductivity:	206 - 228 mS/cm	
pH:	4.9 - 5.3	
Density:	1.092 - 1.112 Kg/L	

50mM Sodium Phosphate, 24mM Citric Acid,

0.92M Ammonium Sulfate, pH 5.1

Unit Description:	Op. ~~Toyopearl~~ TOYOPEARL® 650M Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	13.4 g/L
	Citric Acid, Monohydrate	5.04 g/L
	Ammonium Sulfate	121.6 g/L
pH Adjustment:	NaOH or HCl	TBD
Conductivity:	156 - 173 mS/cm	
pH:	4.9 - 5.3	
Density:	1.068 - 1.084 g/L	

50mM Sodium Phosphate, 24mM Citric Acid,

2.8M Ammonium Sulfate, pH 4.5

Unit Description:	Op ~~Toyopearl~~ TOYOPEARL® 650M Load Dilution	
Component and Concentration:	Sodium Phosphate, Dibasic	13.4 g/L
	Citric Acid, Monohydrate	5.04 g/L
	Ammonium Sulfate	370 g/L

Please replace the paragraph on page 39, lines 2-12 with the following:

Biochemical characterization verified the identity of the purified protein as human ANGIOSTATIN® and indicated that the protein was over 95% pure. The initial step in purification, hydrophobic interaction chromatography (HIC), removed the majority of pigments and extraneous proteins; yielding 80-90% pure ~~ANGIOSTATIN©~~ ANGIOSTATIN®. A number of resins and buffer systems were examined for ~~ANGIOSTATIN©~~ ANGIOSTATIN® binding capacity and specificity. The binding capacity of ~~ANGIOSTATIN©~~ ANGIOSTATIN® protein to ~~Toyopearl~~ TOYOPEARL® Phenyl 650m (TosoHaas) was 20-30% higher than the binding capacity of Phenyl ~~Sepharose~~ SEPHAROSE® high sub (Pharmacia) in PBS containing 3.0 M NaCI [pH 7.4]. At pH 7.4, a buffer system utilizing sodium chloride dramatically increased the binding specificity of Phenyl 650m for ~~ANGIOSTATIN©~~ ANGIOSTATIN® versus an Ammonium Sulfate buffer system.